

## Chapter 3

# Chemical Mutagenesis and Chimera Dissolution in Vegetatively Propagated Banana

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**Abstract** Random mutagenesis has been widely used for forward-genetics and crop breeding since the application of ionising radiation on cereals described in the late 1920s. The development of high-throughput and accurate mutation discovery technologies has enabled reverse-genetic screening of mutant populations in the twenty-first century. The majority of mutation-based approaches in crops have involved seed-propagated species. Large bodies of data are available on the spectrum and density of induced mutations for some mutagens. It is well established that genetic chimerism caused by random accumulation of different mutations in different cells is resolved by sexual propagation and that by the second-generation post-mutagenesis (termed the  $M_2$ ), plants are no longer genetically mosaic. Vegetatively propagated species, however, are quite different as they primarily undergo mitotic propagation. In the absence of meiosis, procedures must be implemented to remove mosaicism and generate plant material that is genotypically homogeneous and suitable for forward- and reverse-genetic screening and breeding. We have previously developed a Targeting Induced Local Lesions IN Genomes (TILLING) platform for the vegetatively propagated triploid banana to investigate the density and spectrum of induced mutations and mechanisms by which tissue culture materials become genotypically homogeneous. Here we provide a detailed protocol for meristematic isolation, mutation induction and dissolution of chimeric sectors focusing on the use of chemical mutagen ethyl methanesulfonate (EMS).

**Keywords** Chemical mutagenesis • *Musa* • Mutation stability • Diplontic selection • Somatic genome • Polyploidy

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J. Jankowicz-Cieslak et al. (eds.), *Biotechnologies for Plant Mutation Breeding*,  
DOI 10.1007/978-3-319-45021-6\_3

### 3.1 Introduction

The nutrition of a billion people relies on crops which are not propagated through seeds, particularly in tropical and Neotropical regions of Africa, Asia, Latin America and the Caribbean. Plants that have completely lost the ability for sexual propagation are called obligate, while those for which meiosis is still possible are called facultative vegetatively propagated plants (van Harten 1998). In a natural setting, genetic diversity is increased in vegetatively propagated crops via the accumulation of spontaneous mutations. Researchers and breeders can further alter the genetics of vegetatively propagated crops through methods including inducing mutations, transgenics, cell fusion hybrids and various genome-editing approaches (Jankowicz-Cieslak and Till 2015). Inducing mutations in combination with other methods such as *in vitro* techniques is advantageous for the improvement and diversification of vegetatively propagated germplasm. This is because recombination and sexual assortment of characters cannot be achieved in mitotically propagated crops, and so inducing mutations is a major way to introduce novel diversity. Moreover, new developments in the field of molecular biology can enable a fast and accurate identification of changes on the genomic level allowing a more precise use of induced mutations.

There are several constraints when working with vegetatively propagated material. The first difficulty appears while performing the mutagenesis experiments. Mutagenesis of vegetative propagules requires pre-mutagenesis preparation of material and oftentimes extensive post-mutagenesis physical manipulations. In many cases, sterile techniques are required for both pre- and post-treatment handling of the material as well as during the mutation steps. Vegetatively propagated crops are usually heterozygous. Mutagenic treatment may uncover otherwise recessive traits that would not express in homozygous material. However, on account of their asexual character and lack of meiotic sieve (the process in which deleterious alleles can be expunged from a genome through genetic recombination and independent assortment), unwanted background mutations are difficult to remove (van Harten 1998). Another result of the mutagenesis performed on *in vitro* material is the generation of chimeric tissues resulting from the fact that each cell treated with a mutagen will accumulate a different collection of mutations. Genotypic heterogeneity can be lessened through several cycles of vegetative *in vitro* regeneration of the mutagenised vegetative propagule (Roux et al. 2001; Jankowicz-Cieslak et al. 2012). However, this has not been extensively studied on the level of genome sequence.

Producing a mutagenised population suitable for forward- or reverse-genetic studies requires careful consideration of several practical experimental design factors. This includes the genetic structure of the target population, choice of the best mutagen, dosage and pre- and post-treatment handling of material. Mutagens that induce point mutations with high density are desirable for reverse-genetic approaches. Chemical mutagens have a known effect on the DNA sequence and have predominated in reverse-genetic projects. This is in part due to the fact that

chemical mutagens producing point mutations are highly predictive in their effect on protein function and there have been decades of development of technologies for mutation discovery (Ng and Henikoff 2003; Colbert et al. 2001; Tsai et al. 2011). The most commonly used chemical mutagen is ethyl methanesulfonate (EMS) which in many species produces almost exclusively G:C to A:T transition mutations (Kurowska et al. 2011). Other mutagens such as gamma and fast neutron irradiation can produce a broader spectrum of mutations ranging from SNPs and small indels to deletions greater than one million base pairs (Jankowicz-Cieslak and Till 2015). While phenotypic consequences of large indels and rearrangements may be greater, data sets on the spectrum and density of such mutations are currently much smaller than that of EMS, and therefore predictability of optimal population size required to recover desired alleles is much lower.

To date there has been limited success in producing new mutant banana varieties via physical irradiation. Three mutant varieties have been officially registered in the mutant variety database (MVD 2016). All were produced using gamma irradiation. The number is far lower than rice, which is the crop with the most officially released varieties (over 800 in total). The reasons are likely manifold. Many fewer people are actively involved in banana breeding compared to rice. The logistics of banana mutation breeding including tissue culture propagation, chimerism, polyploidy, heterozygosity and field space required to find rare favourable mutations also makes banana less tractable than rice. One other possibility may be that mutations are not easily induced in banana at a high density. This may be specific to specific mutagens or dosages previously used. Compounding this was the lack of tools to accurately count induced mutations so that the mutagenesis treatment could be optimised.

Mutagenesis is usually applied in a manner that produces some level of lethality in the treated population. It is hypothesised that physiological differences underlie the enhanced sensitivity of certain species to lethality caused by alkylating agents (Comai and Henikoff 2006). This may be due either to direct cytotoxic effects or differences in the efficiency of DNA repair (Hoffman et al. 2004). Other factors affect the total number of mutations that accumulate in a plant genome. For example, phenotypic and genotypic studies show a higher accumulation of mutations as ploidy levels increase in wheat (Stadler 1929; Uauy et al. 2009).

Prior to the advent of TILLING, direct measurement of the density and spectrum of induced mutations was not carried out in many plant species. This resulted in sometimes contradictory advice on achieving a well-mutagenised population. For example, a range of different recommendations by various research groups for chemical mutagenesis of banana tissue cultures has been reported (Omar et al. 1989; Bhagwat and Duncan 1998). These relied on post-mutagenesis observations of the level of damages on plant growth. One observable trend is that longer periods of exposure to EMS at low concentrations may be desirable because they produce the least amount of visible damage (Savin et al. 1968). The first evaluation of the genomic effect of mutagen on the vegetatively propagated material was conducted by Jankowicz-Cieslak et al. (2012). Tests of incubation time and EMS concentrations for the optimal production of mutagenic populations in banana were

done using traditional TILLING methods employing enzymatic mismatch cleavage and fragment analysis using fluorescence detection. A spectrum of >99 % G:C to A:T transition changes was reported (Jankowicz-Cieslak et al. 2012). The density of one mutation per 57 kb was higher than previously published diploid plants but lower than tetraploid and hexaploid wheat. The results fit the expectation of mutation accumulation first described in the late 1920s (Stadler 1929). Based on this work, we present here a protocol for EMS mutagenesis of isolated banana shoot tips suitable for forward- and reverse-genetic studies, e.g. TILLING or TILLING by sequencing in vegetatively propagated plants.

## 3.2 Materials

### 3.2.1 Culture Medium (S-27)

1. Thiamine hydrochloride.
2. 6-Benzylaminopurine (BAP).
3. L-Cysteine HCl.
4. Sucrose (grade1).
5. Murashige and Skoog (MS) basal salt with minimal organics (e.g. Sigma Cat. No. M-6899).
6. Tissue culture grade water.
7. Gelling agent (e.g. Gelrite).
8. 0.22 µm cellulose acetate (CA) sterilising, low-binding filters.
9. Vacuum pump.
10. Sterile tubes (50 ml).
11. Analytical balance.
12. Weighing trays.
13. Spatula.
14. Magnetic stir bar.
15. Hot plate.
16. pH meter.
17. NaOH.
18. KOH.
19. HCl.
20. Media dispenser.
21. Culture tubes.
22. Closures for culture tubes.
23. Erlenmeyer flasks.
24. Aluminium foil.

### 3.2.2 *Chemical Toxicity Test*

1. Chemical mutagen laboratory equipped with fume hood and flow bench (*see Notes 1 and 2*).
2. Personal protective equipment (dedicated laboratory coat, eye protection/goggles, shoe protection, nitrile gloves).
3. Hazardous liquid waste receptacle (collection vessels for EMS waste solution).
4. Box for dry hazardous material disposal.
5. High-quality, disease-free in vitro plantlets (25 propagules per treatment).
6. Sterile S-27 liquid culture medium. Dispense 12 ml of the culture medium per flask and autoclave.
7. Dimethyl sulfoxide (DMSO).
8. 10 % (w/v) sodium thiosulfate.
9. Ethyl methanesulphonate (EMS) AR grade, M.W. 124.2.
10. Sterile distilled water.
11. Sterile membrane for filtering EMS solution: 25 mm diameter, 0.2  $\mu\text{m}$  pore size.
12. Syringe.
13. Needle.
14. Petri plates (94 mm and 145 mm diameters).
15. Bottles (100 ml, 500 ml).
16. Beakers (500 ml and 1000 ml).
17. Sieves (metal, 70 mm diameter, 10–100  $\mu\text{m}$  pore size).
18. Forceps.
19. Scalpels.
20. Scalpel blades.
21. Parafilm.
22. Orbital shaker.
23. Disposable pipettes (5 ml, 25 ml).

### 3.2.3 *Calculation of Growth Reduction (GR)*

1. Analytical balance.
2. Sterile Petri plates for weighing in vitro material.
3. Forceps.
4. Sterile S-27 liquid culture medium.
5. Standard spreadsheet software, e.g. Microsoft Excel.

### **3.2.4 Bulk Mutagenesis**

1. All materials as listed in Sect. 3.2.1.
2. High-quality, disease-free in vitro plantlets (e.g. 1000 propagules per treatment).

### **3.2.5 Chimera Dissolution**

1. Tissue culture laboratory equipped with sterile flow benches (equipped with gas).
2. Growth rooms with light and temperature control (light regime 65  $\mu\text{mol}/\text{m}^2/\text{s}$ , e.g. Cool White fluorescent tubes, Philips TLP 36/86; temperature regime of  $22^\circ \pm 2^\circ \text{C}$ ).
3. Sterile S-27 liquid culture medium.
4. Sterile S-27 solid culture medium.
5. Ethanol for surface and tools sterilisation.
6. Forceps.
7. Scalpels.
8. Scalpel blades.
9. Petri plates (94 mm and 145 mm diameters).
10. Orbital shaker.

## **3.3 Methods**

### **3.3.1 Preparation of Liquid Culture Medium**

1. Prepare stock solutions of thiamine (1 mg/ml), BAP (0.23 mg/ml) and L-cysteine (4 g/l).
2. Filter sterilise stock solutions.
3. Dispense into 50 ml batches and freeze ( $-20^\circ \text{C}$ ) for further use. Store the working solution at  $4^\circ \text{C}$ .
4. For 1 l of the liquid culture media, use the following: 4.4 g of Murashige and Skoog basal salt with minimal organics, 40 g sucrose, 10 ml L-cysteine, 20 ml BAP and 1 ml of thiamine stock solutions. Use double distilled water.
5. Place the media on the mixer and let it mix properly.
6. Calibrate the pH meter as per manufacturer instructions.
7. While stirring, adjust medium to pH 5.8 using NaOH, HCl or KOH.
8. Dispense 12 ml of the culture medium per Erlenmeyer flask.
9. Close each Erlenmeyer flask tightly over the top with aluminium foil.
10. Autoclave for 20 min at  $120^\circ \text{C}$ .
11. Allow the medium to cool.

12. Store the medium for up to a week in a cold room.

3.3.2 Preparation of Solid Culture Medium

1. For 1 l of solid medium cook 1.8 g of Gelrite and 40 g sucrose in 400 ml of tissue culture grade water.
2. In a separate beaker containing 400 ml water, mix 4.4 g of Murashige and Skoog basal salt with minimal organics, 40 g sucrose, 10 ml L-cysteine, 20 ml BAP and 1 ml of thiamine stock solutions.
3. Place the media on the mixer and let it mix properly.
4. Calibrate the pH meter as per manufacturer instructions.
5. While stirring, adjust the pH to 5.8 using NaOH, HCl or KOH.
6. Heat the solution.
7. Add cooked Gelrite/sucrose and continue stirring and heating until the solution is homogenous.
8. Dispense 8 ml of the medium into culture tubes.
9. Sterilise the medium for 20 min at 120 °C.
10. Allow medium to cool prior to use.
11. Store for up to a week in a cold room.

3.3.3 Chemical Toxicity Test

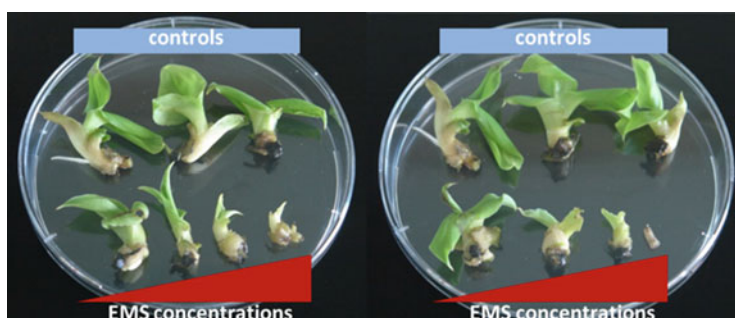
1. Review safety procedures of the chemical mutagenesis laboratory (*see Note 2*).
2. Autoclave all non-disposable materials (e.g. sieves, forceps).
3. Choose appropriate concentrations of EMS solution and incubation times. Examples of dilutions for EMS concentrations are given in Table 3.1.
4. Dispense required volumes of distilled water and autoclave at 120 °C for 15 min. Let the mixture cool to room temperature.
5. Add DMSO using sterile pipette tip.

**Table 3.1** Concentrations chosen for the toxicity test in banana (for two incubation times at 2 and 4 h)

EMS concentration	H <sub>2</sub> O	DMSO	EMS
10 % (stock solution)	88 ml	2 ml	10 ml <sup>a</sup>
0.25 %	95.5 ml	2 ml	2.5 ml <sup>b</sup>
0.5 %	93 ml	2 ml	5 ml <sup>b</sup>
1 %	88 ml	2 ml	10 ml <sup>b</sup>
1.5 %	83 ml	2 ml	15 ml <sup>b</sup>
0 % control	98 ml	2 ml	–
0 % control ( <i>without</i> DMSO)	100 ml	–	–

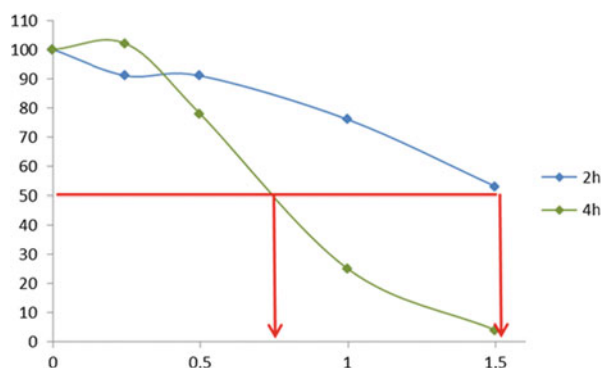
<sup>a</sup>Prepare fresh immediately before experiment  
<sup>b</sup>Prepare fresh from the 10 % EMS stock solution before experiment

6. Prepare meristematic cuttings of banana plantlets (e.g. 25 propagules per treatment including controls). Store in a sealed Petri plate until processed.
7. Prepare a fresh 10 % EMS stock solution by adding the required volume of EMS solution to the sterile water-DMSO mixture. Use a sterile syringe and a 0.2  $\mu$ m filter for this step. Place syringe and filter into a beaker containing 100 mM sodium thiosulfate to inactivate EMS before placing in hazardous waste.
8. Prepare the concentration series of EMS using 10 % EMS stock solution (*see* Table 3.1). Commence with the lowest concentration. Shake the solution vigorously (*see* **Note 3**). Decant approximately equal volumes into each bottle labelled with the appropriate treatment code (concentration and incubation time). This step and any further steps must be carried out in a laminar flow cabinet.
9. Place meristematic cuttings (e.g. 25 each) into each treatment bottle. Place closed bottles on a rotary shaker set at 180 rpm and start incubation. Examples in this protocol are provided for incubation times of 2 and 4 h (*see* Figs. 3.1 and 3.2 and Table 3.2).



**Fig. 3.1** Example of chemical toxicity test performed on the *in vitro* banana material. Grand Naine plantlets were exposed to four concentrations of EMS (from left to right: 0.25 %, 0.5 %, 1 % and 1.5 % EMS) and controls (from left to right: water, DMSO and untreated) and incubated for 2 (*left panel*) and 4 (*right panel*) hours. Material is shown after 30-day post-treatment growth in S-27 liquid media

**Fig. 3.2** Reduction in fresh weight of mutagenised *in vitro* plantlets of Grand Naine as a percentage of the control (*Y axis*) against EMS concentrations (*X axis*). Incubation times of 2 and 4 h are presented. *Red bars* show estimation of mutagen concentrations where 50 % growth reduction is achieved





**Table 3.2** Average fresh weights of 30-day-old Grand Naine in vitro plantlets exposed to varying concentrations of EMS for 2- and 4-h incubation periods

	2-h incubation time		4-h incubation time	
	<sup>a</sup> Fresh weight (g)	Percentage of control (%)	<sup>a</sup> Fresh weight (g)	Percentage of control (%)
0 TIME	1.60	100	1.60	100
0.25 % EMS	1.46	91	1.63	102
0.5 % EMS	1.46	91	1.25	78
1 % EMS	1.21	76	0.40	25
1.5 % EMS	0.84	53	0.07	4

<sup>a</sup>Average of 25 plantlets weighed separately under sterile conditions

10. After the incubation time, quickly but carefully decant each of the treatment batches and rinse with sterile water (approximately 1 l of dH<sub>2</sub>O per treatment).
11. Collect all the liquid waste in a dedicated bucket labelled as hazardous waste (*see Note 4*). Dispose of toxic waste according to local regulations.
12. Transfer explants into Erlenmeyer flasks containing fresh MS medium (five explants per flask) and incubate in the growth chamber for 24 h.
13. After 24 h transfer explants into fresh MS medium to remove any residual EMS and DMSO.
14. Incubate material in the liquid media for the next 30 days (rotary shaker 60 rpm with a continuous light 65 μmol/m<sup>2</sup>/s; Cool White fluorescent tubes, Philips TLP 36/86 and the temperature of 22° ±2 °C).

**3.3.4    Calculation of Growth Reduction (GR)**

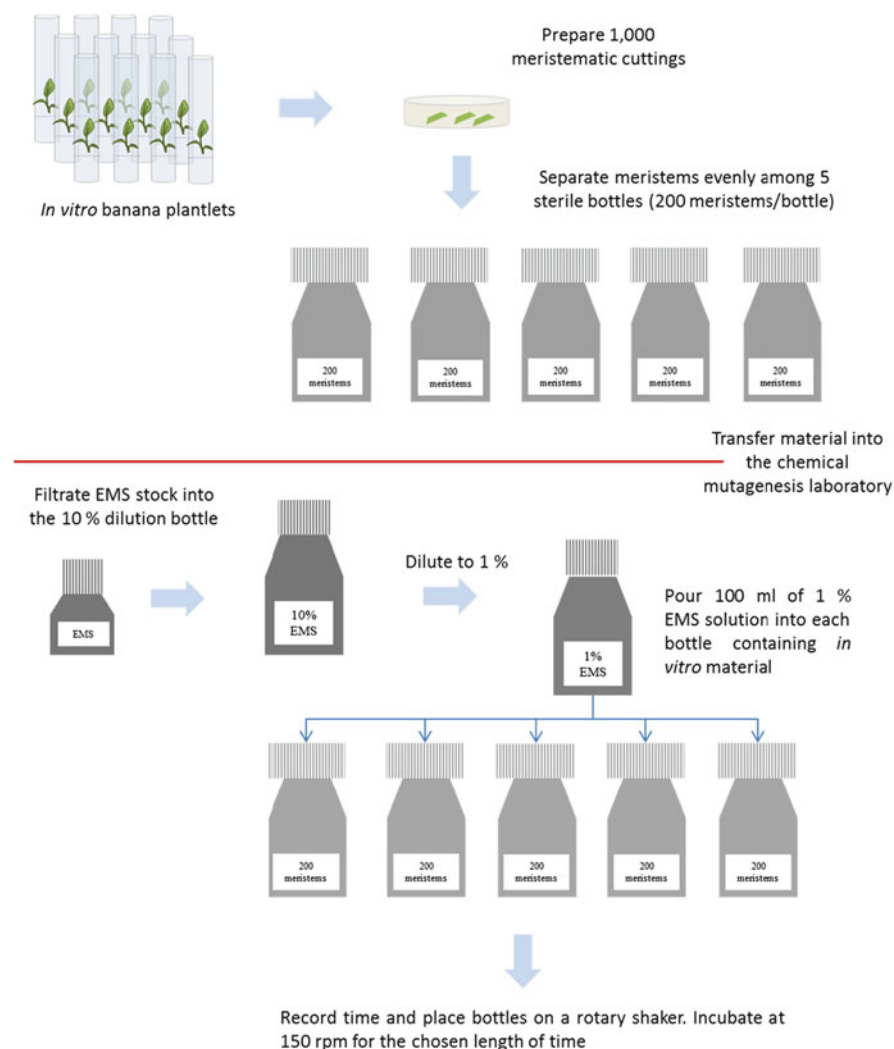
1. After 30 days of incubation, assess the viability of plants by counting surviving plantlets and measuring the fresh weight of each plant (*see Fig. 3.1*).
2. Place a balance in the laminar bench and weigh each mutated plant separately in a sterile Petri plate.
3. Record the data for each treatment and enter it into a spreadsheet (e.g. Microsoft Excel).
4. Calculate average fresh weights for each treatment and for the control (*see Table 3.2*).
5. Calculate percentage of the fresh weight of the plant in relation to the control (*see Note 5*).
6. Plot percentage of control against mutagenesis treatment (*see Fig. 3.2*).
7. Estimate the mutagen concentration required to obtain 50 % of control (*see Fig. 3.2*, red line).
8. Identify concentrations suitable for bulk mutagenesis of your material.

### 3.3.5 Bulk Mutagenesis

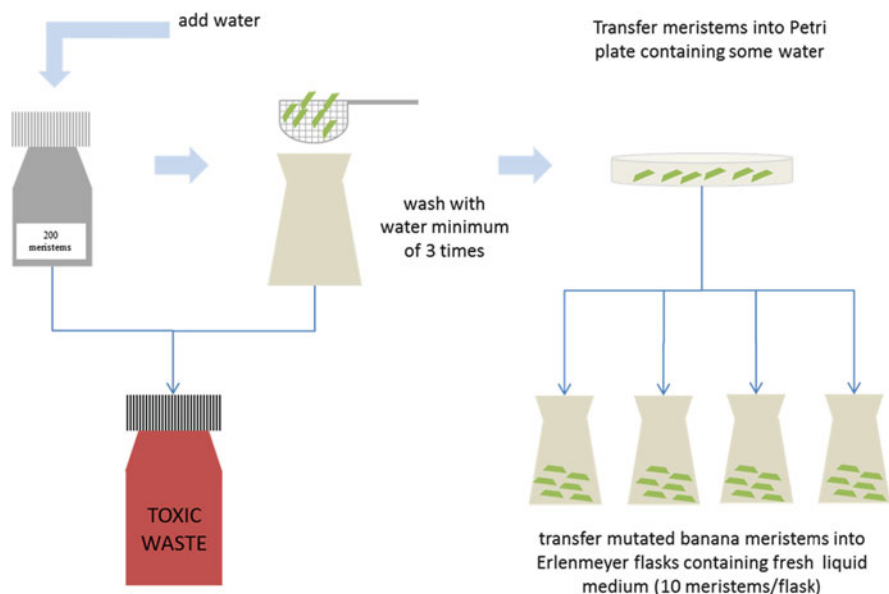
1. Autoclave all non-disposable materials (e.g. sieves, forceps).
2. Choose appropriate EMS concentration and incubation time based on the results obtained from the chemical toxicity test.
3. See Figs. 3.3 and 3.4 for an overview of the bulk mutagenesis procedure.
4. Dispense required volumes of distilled water and autoclave at 120 °C for 15 min. Let the mixture to cool to room temperature.
5. Add DMSO using sterile pipette tip.
6. Prepare meristematic cuttings (e.g. 1000) per each treatment chosen (see **Note 6**).
7. Place a maximum of 200 cuttings into each autoclaved bottle (labelled with EMS concentration and incubation time).
8. Transfer closed bottles containing in vitro material into the chemical mutagenesis laboratory. Add 100 ml of appropriate EMS mixture into each bottle (see **Note 7**).
9. Place bottles on a rotary shaker and incubate at 180 rpm for the chosen length of time.
10. After incubation, add water into the bottles, mix gently and immediately decant carefully using a sterile sieve. See Fig. 3.4 and **Note 8** on post-mutagenesis procedures.
11. Repeat washing step three times.
12. Transfer micropropagules into Erlenmeyer flasks containing liquid medium (maximum of ten meristematic cuttings per flask).
13. Transfer flasks into the growth room and place on a rotary shaker (60 rpm) with continuous light (65  $\mu\text{mol}/\text{m}^2/\text{s}$ ; Cool White fluorescent tubes, Philips TLP 36/86) and the temperature of  $22^\circ \pm 2^\circ \text{C}$ .
14. The next day transfer all treated plantlets into fresh liquid medium.
15. Transfer cultures weekly into fresh liquid media to reduce possible accumulation of phenolic compounds due to the stress caused by mutagenesis.
16. After 30 days of incubation, count survival rates and make note of any visible morphological abnormalities.

### 3.3.6 Chimera Dissolution

1. Grow explants in liquid culture media under constant horizontal rotation at 60 rpm with continuous light (65  $\mu\text{mol}/\text{m}^2/\text{s}$ ; Cool White fluorescent tubes, Philips TLP 36/86) at  $22^\circ \pm 2^\circ \text{C}$ .
2. After 30 days, calculate survival rates of the mutagenised population.
3. Define each mutated meristem as a source of an individual line (see **Note 9**).
4. Isolate meristematic tips from each of mutated plants.

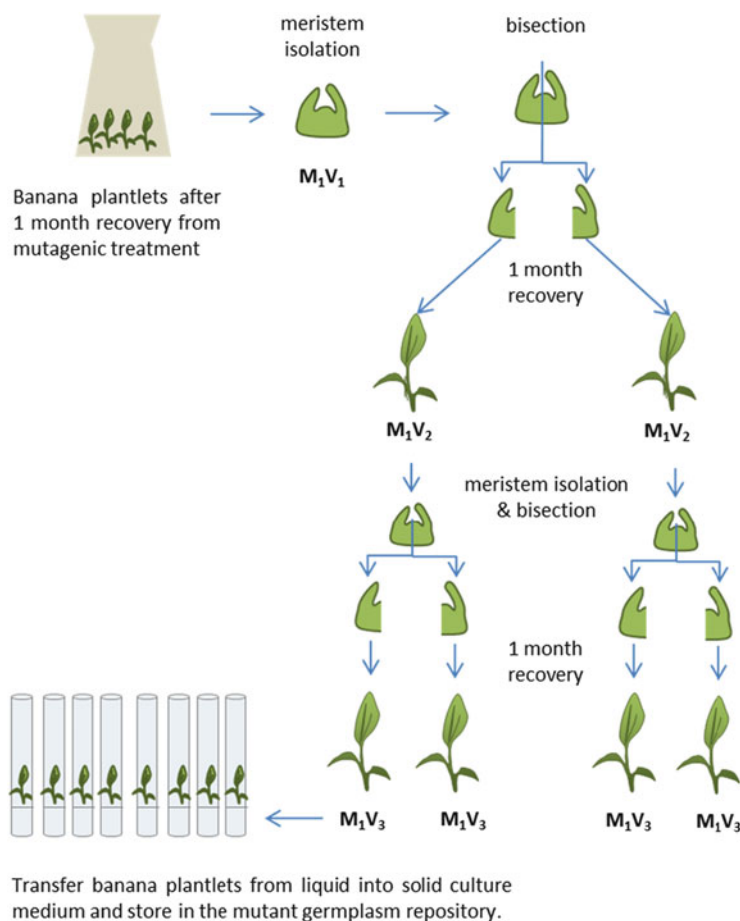


**Fig. 3.3** Bulk mutagenesis process of 1000 *in vitro* meristematic cuttings. Cuttings are first placed into bottles under sterile conditions. Bottles are then transferred to the chemical mutagenesis laboratory equipped with a laminar flow bench. Sterile water + DMSO solutions are prepared. Following this a 10 % EMS stock is freshly prepared in water/DMSO. This is used to make further dilutions of EMS concentration. A subset of cuttings is then added to different EMS concentrations. Both EMS concentration and incubation times are recorded. In this example, material is subjected to 3-h incubation time



**Fig. 3.4** Post-mutagenesis washing of banana *in vitro* meristematic cuttings. Meristems must be carefully washed after EMS treatment in order to remove the residual EMS. After a minimum of three washes are complete, cuttings are placed on Petri plates, sealed with parafilm and moved back into the *in vitro* laboratory. Mutated banana explants should be immediately transferred into fresh liquid medium. Attention is paid to avoid EMS contamination of laboratory

5. Transfer every single isolated meristem into a separate Erlenmeyer flask containing fresh liquid media.
6. In order to remove potential chimeric sectors, propagate shoot tip meristems through longitudinal division into two propagules using a scalpel (*see* Fig. 3.5).
7. Assign a unique number to every flask. This number corresponds to the mutant line that will be generated from this particular individual meristem.
8. Incubate cultures for 4–5 weeks.
9. Repeat the process in order to make the population of  $M_1V_3$  individuals (*see* Fig. 3.5 and **Notes 10 and 11**).
10. At each meristematic division, transfer the material into fresh culture media. Remember to follow the nomenclature assigned for each line.
11. Transfer plants into the solid media for a long-term storage (culture media supplemented by 1.8 % Gelrite).
12. Maintain cultures at  $22^\circ \pm 2^\circ$  under stationary incubation and 12-h light cycle for the duration of the study.
13. Screen mutagenised population using reverse- or forward-genetic methods (*see* **Note 12**).



**Fig. 3.5** Dissolution of chimeras in bananas. After the mutagenic treatment, plants are allowed to grow for 30 days in a liquid culture media at a constant rotary shaking (60 rpm). Each mutated plant is given a unique line number and assigned a population stage starting with  $M_1V_1$ . Chimeras may exist after 30 days recovery period if surviving meristematic cells harbour different mutations. In order to dissolve potential chimeras, meristems are isolated and divided into two parts through a longitudinal cut which results in most cases in generation of two daughter plants. These are allowed to recover and grow for another 30 days. The procedure can be repeated up to three times. At the  $M_1V_3$  plants are transferred into the solid culture media for long-term storage. Maintain cultures at  $22^\circ \pm 2^\circ \text{C}$  under stationary incubation and 12-h light cycle for the duration of the study

### 3.4 Notes

1. All the mutagenesis experiments should be conducted in a dedicated chemical mutagenesis laboratory equipped with a ducted fume hood and toxic waste disposal and should have decontamination procedures.

2. Read the material safety data sheet (MSDS) of materials being used and follow the recommendation of the manufacturer. Pay careful attention to the information for EMS and what to do in case of exposure. It is very important to wear personal protective equipment (gloves must be compatible with chemical mutagens, for instance, PVC or neoprene gloves): safety glasses with side shields or chemical goggles, lab coat, closed-toe shoes, shoe protections and full-length trousers. A double-glove system is advised. EMS can be inactivated by treatment with sodium thiosulfate. Keep beakers of sodium thiosulfate (100 mM) on hand during laboratory procedures to inactivate any spills and to clean tips and other consumables prior to disposing in hazardous waste.
3. EMS is immiscible in water. DMSO is added as a carrier agent to improve miscibility. Thorough shaking of the liquid mixture also facilitates this. Prior to shaking, test bottles and caps with water to ensure there is no leakage. Shake the mixture up and down vigorously approximately 20–30 times.
4. Care should be taken not to splash liquid when decanting. Choose a bucket or beaker and practise first with water. It is also possible to decant directly into a beaker containing 100 mM sodium thiosulfate. This inactivates the EMS prior to disposal in toxic chemical waste container.
5. This calculation is made by dividing the weight of the mutated material (numerator) by the weight of the control material measured at the same time (denominator) multiplied by 100. For example, the weight of material treated for 2 h at 0.25 % is 1.46 g. The control material is 1.6 g. The percentage is then  $1.46/1.60 \times 100 = 91.25 \%$ .
6. *Caution:* If this is being prepared a day before, place in vitro cuttings in a Petri plate with sufficient water. Seal Petri plate with parafilm. The optimal population size depends on the spectrum and density of induced mutations and to a lesser extent on the application (forward- or reverse-genetics). We describe a population size of 1000 because this is easily accommodated in medium size laboratories, and for polyploid bananas, this may be considered suitable in reverse-genetic approaches to recover mutations in most target genes (Jankowicz-Cieslak et al. 2012). For forward-genetic screens, a larger population size may be required because the chance of uncovering useful traits in polyploids in the  $M_1$  generation is reduced due to heterozygous state of induced mutation.
7. *Caution:* Conduct all the steps under the fume hood.
8. *Caution:* The washed out solution is still highly mutagenic and must be carefully discarded following the toxic waste disposal rules and regulations of the respective institution.
9. Ideally a line would represent a pedigree of clonally related material that begins with the first non-chimeric individual such that all subsequent material produced from this progenitor inherits the same mutations. Because the exact state where mutant in vitro plantlets are no longer chimeric is not easily determined, defining a line this way is not always possible. We therefore suggest defining a line simply as the pedigree of all material resulting from a single progenitor-mutated plantlet. Materials in a line therefore may inherit the same mutations as

was observed in Jankowicz-Cieslak et al. (2012) but may also inherit different mutations if chimerism exists in meristematic cells at the time the progenitor is subcultured to produce daughter plantlets.

10. The first population after mutagenic treatment is referred to as  $M_1V_1$  whereby  $V_1$  signifies the first vegetative generation after mutagenic treatment. Increasing numbers following V represent successive vegetative generations, and increasing numbers after M indicate meiotic propagation. This allows tracking of generations in both facultative and obligate vegetatively propagated species.
11. We recommend three subculturing cycles to ensure the dissolution of chimeric materials. If too few cycles are performed, the resulting plants may still be chimeric, and mutations may not be stably passed from one to another vegetative generation. Previous reports suggest that plants may be free of chimeras as early as  $M_1V_2$  (Jankowicz-Cieslak et al. 2012). We recommend that extra propagation cycles after plants are no longer chimeric should be avoided. If done, extra work is performed but no additional genetic variation is produced (Jankowicz-Cieslak et al. 2012).
12. Procedures for TILLING in banana were described in Jankowicz-Cieslak et al. (2012). Tests will need to be performed to determine the degree of clonal relationship within each line. Throughput can be increased with different mutation discovery platforms (Tsai et al. 2011). However, care should be taken when performing three-dimensional pooling as the same mutation may be present in more than one individual making assignment of mutations to individuals difficult. Forward-genetic (phenotypic) screens can also be considered.

**Acknowledgements** The authors wish to thank Dr. Rownak Afza for her guidance and advice on mutagenesis procedures and in vitro methods. We would also like to thank Mr. Andreas Draganitsch for his consistent and highly dedicated support in the tissue culture laboratory. Funding for this work was provided by the Food and Agriculture Organization of the United Nations and the International Atomic Energy Agency through their Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture. This work is part of IAEA Coordinated Research Project D24012.

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